



-2- \* -

C12N15/74 20A

THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification :  
C12N 15/74, 15/48, A61K 39/04  
A61K 39/21, C12N 1/21  
// (C12N 1/21, C12R 1/32)  
(C12N 15/74, C12R 1/32)

A1

(11) International Publication Number:

WO 90/15873

(43) International Publication Date:

27 December 1990 (27.12.90)

(21) International Application Number: PCT/US90/03451

(22) International Filing Date: 18 June 1990 (18.06.90)

(30) Priority data:  
367,894 19 June 1989 (19.06.89) US

(71) Applicant: WHITEHEAD INSTITUTE FOR BIOMEDI-  
CAL RESEARCH [US/US]; Nine Cambridge Center,  
Cambridge, MA 02142 (US).

(72) Inventors: YOUNG, Richard, A. ; ALDOVINI, Anna ; 5  
Saw Mill Brook Road, Winchester, MA 01890 (US).

(74) Agents: GRANAHAH, Patricia et al.; Hamilton, Brook,  
Smith & Reynolds, Two Militia Drive, Lexington, MA  
02173 (US).

(81) Designated States: AT (European patent), AU, BE (Euro-  
pean patent), CA, CH (European patent), DE (Euro-  
pean patent)\*, DK (European patent), ES (European pa-  
tent), FR (European patent), GB (European patent), IT  
(European patent), JP, LU (European patent), NL (Eu-  
ropean patent), SE (European patent).

Published

*With international search report.*

*Before the expiration of the time limit for amending the  
claims and to be republished in the event of the receipt of  
amendments.*

(54) Title: VECTOR-MEDIATED GENOMIC INSERTION AND EXPRESSION OF DNA IN BCG

(57) Abstract

Recombinant integration vectors useful for introduction of DNA of interest into mycobacteria are disclosed. The integra-  
tion vector includes: 1) DNA sequences homologous to sequences present in the mycobacterial genome; 2) DNA encoding a pol-  
ypeptide or protein of interest; 3) DNA encoding a selectable marker of mycobacterial origin; and 4) DNA encoding a regulated  
mycobacterial promoter region. The present invention also relates to recombinant mycobacteria useful for administration to  
mammalian hosts for purposes of immunization.

AN

### DESIGNATIONS OF "DE"

Until further notice, any designation of "DE" in any international application whose international filing date is prior to October 3, 1990, shall have effect in the territory of the Federal Republic of Germany with the exception of the territory of the former German Democratic Republic.

#### FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

|    |                              |    |                                       |    |                          |
|----|------------------------------|----|---------------------------------------|----|--------------------------|
| AT | Austria                      | ES | Spain                                 | MC | Monaco                   |
| AU | Australia                    | FI | Finland                               | MG | Madagascar               |
| BB | Barbados                     | FR | France                                | ML | Mali                     |
| BE | Belgium                      | GA | Gabon                                 | MR | Mauritania               |
| BF | Burkina Faso                 | GB | United Kingdom                        | MW | Malawi                   |
| BG | Bulgaria                     | GR | Greece                                | NL | Netherlands              |
| BJ | Benin                        | HU | Hungary                               | NO | Norway                   |
| BR | Brazil                       | IT | Italy                                 | RO | Romania                  |
| CA | Canada                       | JP | Japan                                 | SD | Sudan                    |
| CF | Central African Republic     | KP | Democratic People's Republic of Korea | SE | Sweden                   |
| CG | Congo                        | KR | Republic of Korea                     | SN | Senegal                  |
| CH | Switzerland                  | LI | Liechtenstein                         | SU | Soviet Union             |
| CM | Cameroon                     | LK | Sri Lanka                             | TD | Chad                     |
| DE | Germany, Federal Republic of | LU | Luxembourg                            | TC | Togo                     |
| DK | Denmark                      |    |                                       | US | United States of America |

-1-

VECTOR-MEDIATED GENOMIC INSERTION AND  
EXPRESSION OF DNA IN BCG

Description

Background

Mycobacteria represent major pathogens of man and  
05 animals. For example, tuberculosis is generally caused  
in humans by Mycobacterium (M.) tuberculosis and in  
cattle by Mycobacterium (M.) bovis (which can be

transmitted to humans and other animals, in whom it causes tuberculosis). Tuberculosis remains widespread and is an important public health problem, particularly in developing countries. It is estimated that there are approximately 10 million cases of tuberculosis worldwide, with an annual mortality of 3 million. Joint International Union Against Tuberculosis and World Health Organization Study Group, Tubercle, 63:157-169 (1982).

Leprosy, which is caused by M. leprae, afflicts over 10 million people, primarily in developing countries. Bloom, B. R. and T. Godal, Review of Infectious Diseases, 5:657-679 (1984). M. tuberculosis and mycobacteria of the avium-intracellulare-scrofulaceum (MAIS) group represent major opportunistic pathogens of patients with acquired immunodeficiency disease (AIDS). Centers for Disease Control, Morbidity and Mortality Weekly Report, 34:774 (1986). M. pseudotuberculosis is a major pathogen of cattle.

On the other hand, Bacille Calmette-Guerin (BCG), an avirulent strain of M. bovis, is the most widely used human vaccine in the world and has been used as a live vaccine for more than 50 years. In the past 35 years, it has been administered to over 2.5 billion people, with remarkably few adverse effects (e. g., estimated mortality of 60/billion). BCG has been found in numerous studies to have protective efficacy against tuberculosis. However, it was found not to be effective in preventing pulmonary tuberculosis in some trials, for example in Southern India. Tuberculosis Prevention Trial, Madras, Indian Journal of Medical Research, 72 (suppl.):1-74 (1980).

Mycobacteria have been proposed for use as live vaccine vehicles. Genes encoding antigens from a variety

05 of pathogens can be expressed in recombinant mycobacteria  
such that the adjuvant properties of the mycobacteria  
stimulate an immune response to the foreign antigens  
which, in turn, provide protective immunity against these  
pathogens. Mycobacteria offer several advantages as  
vaccine vehicles, including a long record of use in  
humans with a very low incidence of complications,  
stability in the field and single dose usage. The  
ability to manipulate the mycobacterial genome is  
10 essential to developing this candidate vaccine vehicle.

The ideal molecular genetic system includes a  
transformation method, vectors that can be manipulated in  
vitro, selectable nutritional and drug markers and an  
ability to replace specific genomic DNA sequences with  
15 exogenous sequences. A means by which exogenous DNA  
encoding a protein of interest can be introduced into the  
mycobacterial genome in place of specific genomic DNA  
sequences to produce a recombinant mycobacterium capable  
of expressing the encoded protein would be very useful.

## 20 Summary of the Invention

The present invention relates to marked target  
strains of mycobacteria; to genetically recom-  
binant (genetically engineered) cultivable mycobacteria  
which express DNA of interest integrated into the  
mycobacterial genome and expressed under the control of a  
25 regulated promoter region; to integration vectors useful  
for the introduction of DNA of interest and a regulated  
promoter region into the mycobacterial genome; to a  
vector-mediated method of integrating DNA of interest and  
30 a regulated promoter region stably into the mycobacterial  
genome, to produce genetically recombinant mycobacteria;  
and to expression or vaccine vehicles which are such

recombinant mycobacteria capable of expressing the integrated DNA.

05 The integration vector of the present invention has made it possible to integrate DNA of interest into the genome of mycobacteria, such as Mycobacterium smegmatis (M. smegmatis) and Mycobacterium bovis-BCG (BCG). The integration vector includes an expression cassette, which is introduced into the mycobacterial genome by site-specific double homologous recombination. The  
10 expression cassette includes DNA of interest and a regulated promoter region; the two components are in such proximity to one another that expression of the DNA of interest in the recombinant mycobacterium is controlled by the regulated promoter region. The regulated promoter region is generally a mycobacterial promoter region, but  
15 can also be a regulated bacterial promoter, such as the E. coli lacZ promoter. For example, a heat shock protein promoter region or stress protein promoter region (e.g., hsp70, hsp60) can be inserted into a recombinant plasmid vector in close proximity to the DNA of interest, such  
20 that translation of the gene is controlled by the mycobacterial promoter region and ribosome binding sites. DNA of interest is from a source other than the mycobacterium into which it is being integrated and is  
25 all or a portion of a gene or genes encoding protein(s) or polypeptide(s) of interest. DNA of interest introduced in this manner is referred to as integrated DNA or integrated DNA of interest. The proteins or polypeptides of interest can be, for example, proteins or  
30 polypeptides against which an immune response is desired (antigen(s) of interest), enzymes, lymphokines, immunopotentiators, and reporter molecules of interest in a diagnostic context.

-5-

05 In the method of producing recombinant mycobacteria  
of the present invention, double homologous recombination  
occurs between plasmid-borne DNA sequences homologous to  
DNA sequences in the mycobacterial genome and the homolo-  
gous mycobacterial sequences. As a result of the recom-  
bination event, a recombinant mycobacterium is produced  
which is very similar to the corresponding unmodified  
mycobacterium. The recombinant mycobacteria includes the  
expression cassette (i.e., DNA of interest and the  
10 regulated promoter region), which is not present in the  
unmodified mycobacterium. Unlike recombinant  
mycobacteria produced by other methods, those produced by  
the present vector-mediated method do not contain vector  
DNA sequences or other sequences not normally present in  
15 mycobacteria other than those of the DNA of interest.

The resulting recombinant mycobacteria (e.g.,  
recombinant BCG, recombinant M. smegmatis) are  
particularly useful as vehicles in which the DNA of  
interest can be expressed. Such vehicles can be used,  
20 for example, as regulatable vaccine vehicles which  
express a polypeptide or a protein of interest (or more  
than one polypeptide or protein), such as an antigen or  
antigens, for one or more pathogens of interest.

The recombinant mycobacteria can also be used as a  
25 vehicle for expression of immunopotentiators, enzymes,  
pharmacologic agents and antitumor agents; for expression  
of a polypeptide or a protein useful in producing an  
anti-fertility vaccine vehicle; or for expression of  
stress proteins, which can be administered to evoke an  
30 immune response or to induce tolerance in an autoimmune  
disease (e.g., rheumatoid arthritis). Recombinant  
mycobacteria can, for example, express protein(s) or  
polypeptide(s) which are growth inhibitors or are  
cytotoxic for tumor cells (e.g., interferon  $\alpha$ ,  $\beta$  or  $\gamma$ ;

interleukins 1-7, tumor necrosis factor (TNF)  $\alpha$  or  $\beta$ ) and, thus, provide the basis for a new strategy for treating certain human cancers (e.g., bladder cancer, melanomas). Pathogens of interest include any virus, microorganism, or other organism or substance (e.g., a toxin or toxoid) which causes disease. The present invention also relates to methods of vaccinating a host with the recombinant mycobacterium to elicit protective immunity in the host. The recombinant vaccine can be used to produce humoral antibody immunity, cellular immunity (including helper and cytotoxic immunity) and/or mucosal or secretory immunity. In addition, the present invention relates to use of the antigens expressed by the recombinant cultivable mycobacterium as vaccines or as diagnostic reagents.

The vaccine of the subject invention has important advantages over presently-available vaccines. In particular, the recombinant mycobacteria described herein express DNA of interest as a protein product without extraneous or additional proteins. This differs from presently available vaccine vehicles in which a gene or genes of interest are expressed in a recombinant form (e.g., fusion protein). Further, mycobacteria have adjuvant properties among the best currently known and, thus, stimulate a recipient's immune system to respond to other antigens with great effectiveness. This is a particularly valuable aspect of the vaccine because it induces cell-mediated immunity and will, thus, be especially useful in providing immunity against pathogens in cases where cell-mediated immunity appears to be critical for resistance. Second, the mycobacterium stimulates long-term memory or immunity. As a result, a single (one-time) inoculation can be used to produce



-7-

long-term sensitization to protein antigens. Using the vaccine vehicle of the present invention, it is possible to prime long-lasting T cell memory, which stimulates secondary antibody responses neutralizing to the infectious agent or the toxin. This is useful, for example, against tetanus and diphtheria toxins, pertussis, malaria, influenza, herpes viruses and snake venoms.

BCG in particular has important advantages as a vaccine vehicle in that: 1) it is the only childhood vaccine currently given at birth; 2) in the past 40 years, it has had a very low incidence of adverse effects, when given as a vaccine against tuberculosis; and 3) it can be used repeatedly in an individual (e. g., in multiple forms).

A further advantage of BCG in particular, as well as mycobacteria in general, is the large size of its genome (approximately  $3 \times 10^6$  bp in length). Because the genome is large, it is able to accommodate a large amount of DNA from another source (i.e., DNA of interest) and, thus, can be used to make a multi-vaccine vehicle (i. e., one carrying DNA of interest encoding protective antigens for more than one pathogen).

#### Brief Description of the Drawings

Figure 1 is a schematic representation of the construction of a marked target mycobacterial strain.

Figure 2 is a schematic representation of insertion of DNA of interest into a marked target BCG.

Figure 3 is a schematic representation of vector pY6014, which contains the mycobacterial PyrF gene and flanking DNA sequences.

Figure 4 is a schematic representation of vector pY6015, which contains the aph gene (encodes kanamycin resistance) inserted at the PyrF site of pY6014.

05 Figure 5 is a schematic representation of vector pY6016, which contains the mycobacterial PyrF gene and flanking DNA sequences of pY6014 with several restriction sites removed.

10 Figure 6 is a schematic representation of vector pY6017, which contains the hsp70 gene inserted ~70bp downstream of PyrF in pY6016.

Figure 7 is a schematic representation of vector pY6018, which contains the hsp70 promoter and the HIV1 gag gene inserted in the hsp70 coding site of pY6017.

15 Figure 8 is a schematic representation of vector pY6019, which contains the hsp70 promoter and the HIV1 pol gene inserted in the hsp70 coding site of pY6017.

Figure 9 is a schematic representation of vector pY6020, which contains the hsp70 promoter and the HIV1 env gene inserted in the hsp70 coding site of pY6017.

20 Figure 10 is a schematic representation of vector pY6021, which contains the hsp70 promoter and the SIV1 gag gene inserted in the hsp70 coding site of pY6017.

25 Figure 11 is a schematic representation of vector pY6022, which contains the hsp70 promoter and the SIV1 pol gene inserted in the hsp70 coding site of pY6017.

Figure 12 is a schematic representation of vector pY6023, which contains the hsp70 promoter and the SIV1 env gene inserted in the hsp70 coding site of pY6017.

#### Detailed Description of the Invention

30 Mycobacterium bovis-BCG (BCG or M. bovis-BCG) is an avirulent M. bovis derivative which is widely used

-9-

throughout the world, particularly to provide protection against tuberculosis.

Because M. bovis-BCG has excellent adjuvant activity for induction of cell-mediated immunity, stimulates  
05 long-term memory (immunity) and has a low mortality associated with its use, it is an excellent candidate as a recombinant vehicle for the expression of proteins of interest, such as a protein antigen, enzymes, lymphokines, and immunopotentiators.

10 It is now possible, using the marked target strain, the integration vector and the method of the present invention, to produce recombinant mycobacteria in which DNA of interest and a regulated promoter region are stably integrated and in which the DNA of interest is  
15 expressed under the control of the regulated promoter region. In particular, it is now possible to stably introduce DNA of interest into BCG genomic DNA in combination with a regulated promoter region, such as that of the heat shock protein (hsp)70 or the hsp60 promoter  
20 region. The resulting recombinant BCG has very similar characteristics to those of the unmodified BCG. The recombinant genome includes the DNA of interest and the regulated promoter region, which are not in the unmodified BCG. Expression of the DNA of interest, to  
25 produce the encoded protein or polypeptide, is under the control of the regulated promoter region. The recombinant mycobacteria are useful as expression vehicles, in which expression of the DNA of interest is under the control of the regulated promoter region and,  
30 thus, is regulatable.

An important feature of this system is that the genomic DNA of the recombinant BCG is minimally disrupted. DNA of interest is inserted along with the

regulated promoter region into a site between the PyrF gene and the adjacent gene, so that the expression of the mycobacterial genes is not affected. Thus, modified BCG is essentially the same as the unmodified BCG.

05 The following is a description of construction of marked mycobacterial target strains; construction of  
integration vectors useful for introducing DNA of inter-  
est into marked mycobacterial target strains; production  
of recombinant mycobacteria having DNA of interest stably  
10 integrated into genomic DNA; and uses of the resulting recombinant mycobacteria. Although the following is described in terms of BCG, it is to be understood that the methods and vectors described can also be used to produce recombinant M. smegmatis, as well as other  
15 recombinant mycobacteria, such as: M. avium, M. phlei, M. fortuitum, M. lufu, M. paratuberculosis, M. habana, M. scrofulaceum, M. tuberculosis, and M. intracellulare.

Figure 1 is a schematic representation of construction of a marked mycobacterial (BCG) target  
20 strain, which is the target for further manipulation using standard genetic engineering methods. A recombinant plasmid containing mycobacterial DNA in which the PyrF gene has been replaced with a gene encoding a selectable marker is used to transform M. bovis-BCG,  
25 using standard methods such as electroporation. A double homologous recombination event (indicated in Figure 1 by two Xs) occurs between plasmid-borne sequences homologous to sequences within the mycobacterial genome. For double homologous recombination to occur, plasmid-borne  
30 sequences homologous to sequences in the mycobacterial genome are positioned on either side of the gene encoding the selectable marker. The result is a replacement in the mycobacterial genome of the PyrF gene with the gene

-11-

encoding the selectable marker (e.g., aph as represented in Figure 1). Transformants (i.e., mycobacteria in which the PyrF gene has been replaced by the selectable marker-encoding gene) exhibit a change in phenotype different from the phenotype observed in the unmodified mycobacterium. Transformants are selected on the basis of either of these characteristics.

The resulting marked mycobacterial target strain is used as a target for further manipulation, using known techniques, by which DNA of interest is integrated into mycobacterial genomic DNA. An integration vector such as that represented in Figure 2 is used for this purpose. The integration vector includes DNA sequences homologous to DNA sequences in the target mycobacterial genome, DNA encoding a selectable marker of mycobacterial origin, DNA encoding a protein or polypeptide of interest (DNA of interest) and DNA encoding a regulated mycobacterial promoter region. The remaining sequences present in the integration vector are those of an appropriate plasmid vector (such as pUC19). Construction of integration vectors of the present invention are described in detail below. The integration vector is introduced into the marked mycobacterial target strain using standard techniques (e.g., electroporation). A double homologous recombination event (indicated in Figure 2 by 2 Xs) occurs between plasmid-borne mycobacterial sequences which are homologous to sequences in the target mycobacterial genome and which flank the DNA encoding a selectable marker of mycobacterial origin (on one side) and the DNA of interest (on the other side). The result is a replacement in the marked target strain of the gene encoding a selectable marker (in Figure 2, aph) and flanking sequences with DNA homologous to mycobacterial

sequences, the DNA encoding a selectable marker of mycobacterial origin, the DNA of interest, and DNA encoding a regulated mycobacterial promoter region. This is represented schematically in Figure 2. As shown, the double homologous recombination event results in recombinant mycobacteria which have two phenotypic characteristics useful for identifying and selecting cells containing the DNA of interest and regulated promoter region.

DNA of interest can be of any origin and is DNA which is all or a portion of a gene or genes encoding protein(s) or polypeptide(s) of interest. The term polypeptide of interest, as used herein, includes all or a portion of a protein to be expressed. Such DNA of interest is expressed in the genetically recombinant mycobacteria, in which it is integrated into the mycobacterial genome. The DNA of interest is introduced into the marked mycobacterial target strain as a component of an expression cassette, which also includes a regulated promoter region. As a result of the double homologous recombination by which integration occurs, recombinant mycobacteria contain DNA of interest, the regulated promoter region and components which are equivalents of the corresponding sequences present in the unmodified mycobacteria. For example, as shown in Figure 2, the recombinant BCG differs from the unmodified BCG only by the presence of the DNA of interest and the regulated promoter region. The mycobacterial genomic DNA has been changed (by replacement of the *PyrF* gene and flanking sequences with the *aph* gene and flanking sequences) and then "reconstructed" in such a manner that equivalents of the *PyrF* gene and flanking sequences are integrated, along with the expression cassette. The

-13-

"net" change is incorporation into the mycobacterial genome of DNA of interest and a regulated promoter region.

05 The regulated promoter region present in the expression cassette can be any regulated promoter region functional in mycobacteria. The regulated promoter region includes the transcriptional promoter and translational start site, including the ribosome binding sites. In general, the promoter region is of  
10 mycobacterial origin. However, regulated bacterial promoters, such as the E. coli lacZ promoter, can also be used. In one embodiment of the present invention, the regulated promoter region is a heat shock promoter region (hsp) of mycobacterial origin, such as the BCG hsp70 or  
15 hsp60. Use of such a regulated promoter region provides an important advantage to the recombinant mycobacteria; particularly in expression vehicles which are vaccine vehicles. In circumstances, such as increased temperature or bacterial infection, in which an  
20 individual is stressed, the hsp promoters are turned up and enhanced protein production occurs. In the present invention, this is particularly useful because expression of the DNA of interest is under the control of the regulated promoter region, which is turned up and, thus,  
25 drives expression of the DNA of interest at a higher than normal level.

When DNA of interest is integrated into the mycobacterial genome as a result of a single homologous recombination event, plasmid DNA and the DNA of interest  
30 are integrated into the mycobacterial genome. The resulting recombinant mycobacterium is unstable due to identical mycobacterial sequences in close proximity to one another. As a result, recombination of homologous

-14-

sequences can again occur. This results in "looping out" (resolution), which removes the recombinant plasmid and DNA of interest, thus eliminating the genomic integration. This does not occur in recombinant  
05 mycobacteria of the present invention.

Construction of one marked BCG target strain and of several plasmid vectors and integration vectors, as well as integration of DNA of interest into the marked target strain genomic DNA, are described below with reference to  
10 the figures.

To produce a marked mycobacterial target strain, the mycobacterial *PyrF* gene in the M. bovis-BCG genome was replaced with the *aph* gene, which encodes kanamycin resistance, using a plasmid vector (designated pY6015)  
15 containing the *aph* gene and capable of transforming M. bovis-BCG. The *PyrF* gene encodes orotidine monophosphate decarboxylase, which allows mycobacteria to grow in medium lacking uracil. The normal or wild type BCG is a uracil prototroph (grows in the absence of uracil) and  
20 kanamycin sensitive and has the phenotype ( $URA^+$ ,  $KAN^S$ ). As described in detail in the following sections, and represented in Figures 3 and 4, plasmid pY6015 was constructed by replacing the *PyrF* gene in the vector pY6014, which also contains sequences flanking *PyrF*  
25 (Figure 3), with an *aph* gene (Figure 4). Vector pY6015 contains the *aph* gene flanked by BCG sequences normally found flanking either side of the *PyrF* gene in the BCG genome.

Recombinant plasmid pY6015, was used to transform  
30 mycobacterial cells (e.g., M. bovis-BCG), using standard electroporation techniques, to produce a target BCG strain which is then manipulated, as described below, to stably integrate DNA of interest into its genome.



-15-

"Recognition" of homology between sequences present in the plasmid-borne mycobacterial DNA sequences located on both sides or ends of the gene encoding a selectable marker (here the aph gene) and sequences in the mycobac-  
05 terial genome (PyrF flanking sequences), results in double homologous recombination. As diagrammed in Figure 1, the recombination event resulted in replacement of the mycobacterial PyrF gene and integration of the plasmid-borne aph gene, thus producing a "marked" BCG target  
10 strain having the phenotype (URA<sup>-</sup>, KAN<sup>R</sup>). Electroporated cells were plated onto medium which contained kanamycin and uracil. Only those cells which contained the intact aph gene survived under these conditions.

A second recombinant plasmid, referred to as an  
15 integration vector, was then used to introduce DNA of interest into the marked mycobacterial target cells (here, the mycobacteria containing the aph gene). The integration vector used to transform the target BCG includes: 1) DNA sequences (referred to as plasmid-borne  
20 DNA sequences) homologous to DNA sequences in the target BCG genome, which are necessary for double homologous recombination to occur (between plasmid-borne mycobacterial sequences and DNA sequences in the marked target BCG genome); 2) DNA encoding a selectable marker of  
25 mycobacterial origin; 3) DNA encoding a regulated mycobacterial promoter region to control or drive expression of DNA of interest; and 4) DNA of interest encoding a protein or polypeptide of interest. DNA  
30 encoding a selectable marker of mycobacterial origin is defined herein as nucleotide sequences, isolated from the mycobacterial genome or nucleotide sequences sufficiently similar to the mycobacterial sequences to encode the same selectable or phenotypic characteristics, including DNA

sequences produced by chemical or mechanical synthesis, or by cloning and amplification. The target BCG cells are transformed with the integration vector, using known techniques. The mycobacterial sequences in the  
05 integration vector were the same as those present in the mycobacterial genome. Alternatively, they can be sufficiently similar to those present in the mycobacterial genome to make homologous recombination possible. As represented in Figure 2, a PyrF gene has  
10 been used in combination with DNA of interest encoding a protein or polypeptide of interest. "Recognition" of homology of sequences present in the plasmid-borne mycobacterial DNA (i.e., PyrF DNA sequences and flanking sequences) and identical or sufficiently similar  
15 sequences present in the marked target mycobacterial genome results in double homologous recombination between regions of the incoming (plasmid-borne) mycobacterial DNA and the genomic mycobacterial DNA. The result is integration of the PyrF gene and the expression cassette,  
20 which includes the DNA of interest and the hsp 70 promoter region, into the mycobacterial genome, with the concomittant deletion of the aph gene.

Thus, as described above, it is possible to  
25 integrate into the mycobacterial genome DNA encoding a protein or polypeptide of interest and to identify and select those cells which contain DNA of interest stably integrated into the genome. In addition, such DNA of interest is integrated into the mycobacterial genome at a selected site (i.e., PyrF gene locus). This same  
30 approach can, of course, be used to integrate DNA of interest into other selected sites on mycobacterial genomic DNA. For example, the site chosen can be any gene necessary for cell metabolism. In this case, a site

-17-

on the genome, at which integration is desired, can be selected. An integration vector can be constructed, as described previously. The DNA of interest can be stably integrated into mycobacterial genomic DNA and cells containing the stably integrated DNA of interest selected, in the same manner as described previously.

Until the present time, it has not been possible to produce recombinant mycobacterial expression vehicles in which DNA encoding a polypeptide or protein, such as one against which an immune response is desired, is stably integrated, at selected sites and in selected orientations, in genomic DNA. Further, according to the method of the present invention, the expression of DNA of interest stably integrated into the mycobacterial genome is controlled by a regulated mycobacterial promoter region. For example, a mycobacterial promoter and ribosome binding site, such as a heat shock protein promoter region (e.g., hsp70, hsp60), serve as regulatable expression signals controlling expression of the DNA of interest. As represented in Figure 6, the expression cassette can include a polylinker in sequences surrounding the mycobacterial sequences necessary for double homologous recombination (i.e., the PyrF gene). As a result, DNA of interest can be inserted into the mycobacterial genome and mycobacterial expression signals will control the level of production of the protein or polypeptide of interest. Selection of mycobacterial cells in which the PyrF-expression cassette-DNA of interest combination are stably integrated can be carried out as described previously.

It is now possible, using the method and the integration vector of the present invention, to integrate DNA of interest into a cultivable mycobacterium and

regulate the expression of the DNA of interest. The DNA encoding a polypeptide or protein against which an immune response is sought, which is present in the integration vector, can be obtained by isolation of the naturally-  
05 occurring DNA (e.g., from the pathogenic organism or toxin-producing organism); by cloning and amplification of the DNA sequence of interest, using known genetic engineering techniques (See, for example, Maniatis, T. et. al. Molecular Cloning: A Laboratory Manual, Cold  
10 Spring Harbor, N.Y. (1982).); or by mechanical or chemical synthesis (e.g., polymerase chain reaction (PCR)).

Similarly, plasmid-borne DNA sequences necessary for homologous recombination can be isolated from a source in  
15 which it occurs in nature, produced by means of standard genetic engineering techniques or synthesized chemically or mechanically. The characteristic which serves as the basis for selection of a marked mycobacterial target strain containing the gene encoding a selective marker  
20 can be, as described, drug resistance. The gene can encode, for example, kanamycin resistance, viomycin resistance, thiostrepton resistance, hygromycin resistance or bleomycin resistance. Alternatively, an auxotrophy strategy can be used, such that selection is based  
25 on the ability of mycobacteria in which integration has occurred to survive, when grown on appropriate medium.

The integration vector described above and in the following sections can be used to integrate DNA of  
interest which encodes one or more antigens for one or  
30 more pathogens of interest into the mycobacterial genome. It can also be used, by integrating DNA encoding an appropriate protein, such as human gonadotropin hormone (HGH) fragments, into mycobacteria, to produce an

-19-

anti-fertility "vaccine." These vectors can also be used to integrate DNA encoding a protein or a polypeptide which is a growth inhibitor for or cytotoxic to tumor cells. The resulting recombinant mycobacteria can be used, respectively, to non-specifically augment immune responses to foreign antigens expressed in mycobacteria and to treat some human cancers.

As a result, it is possible to produce recombinant mycobacterial vaccines which can be used to immunize individuals against, for example, leprosy, tuberculosis, malaria, diphtheria, tetanus, leishmania, salmonella, schistosomiasis, measles, mumps, herpes, and influenza. Genes encoding one or more protective antigens for one or more of the disease-causing pathogens can be integrated into the mycobacterial genome. Of particular value is the ability to integrate genes encoding antigens of pathogens which require T-cell memory or effector function. Administration of the resulting recombinant mycobacterial vaccine to a host results in stimulation of the host's immune system to produce a protective immune response.

Construction of a recombinant plasmid vector and  
production of a marked mycobacterial target strain

A marked mycobacterial target strain was produced to be able to introduce and express DNA of interest at a specific M. bovis-BCG gene locus, and to select for recombinant mycobacteria in which integration occurs. For example, the PyrF gene is a particularly useful genetic tool because it permits positive and negative selections and does not suffer from several problems associated with drug resistance markers. For example, spontaneous resistance to many drugs occurs at relatively

high frequencies in bacteria because of the large number of different mutations that can affect cell permeability to drugs. Further, it is often undesirable in potentially pathogenic organisms, or in candidate live vaccines to have a drug resistance marker present in the vaccine vehicle.

In the method of the present invention, a marked mycobacterial target strain is produced in which the normal mycobacterial *PyrF* gene is replaced with a plasmid-borne *aph* gene. A starting plasmid, pY6014, was produced by cloning the entire BCG *PyrF* gene and flanking DNA sequences (~0.5-1.5 kb on either side) into pUC19 (Figure 3). Subsequently, the *PyrF* gene was replaced with the *aph* gene isolated from Tn903 to produce plasmid pY6015. Normal mycobacterial cells are uracil prototrophs ( $URA^+$ ) and kanamycin sensitive ( $KANS^S$ ). The pY6015 vector contains the *aph* gene inserted between the BCG *PyrF* flanking sequences. This vector was used to transform BCG cells to produce a marked mycobacterial target strain (i.e., BCG cells in which the *aph* gene has been inserted into the genome). A double homologous recombination event, indicated in Figure 1 by two Xs, occurs between the plasmid-borne mycobacterial sequences homologous to sequences in the mycobacterial genome (i.e., *PyrF* flanking sequences). The result is a replacement of the mycobacterial *PyrF* gene with the *aph* gene which encodes kanamycin resistance. As a result of the recombination event, the transformants have a distinctive double phenotype. The marked target BCG strain are uracil auxotrophs ( $URA^-$ ) and kanamycin resistant ( $KAN^R$ ). Thus, mycobacteria that have undergone gene replacement can be directly selected by plating transformants on medium containing kanamycin and uracil,

-21-

or alternatively, on medium containing 5-fluoro-orotic acid (FOA) and uracil (the loss of the PyrF gene produces cells that are resistant to FOA).

Construction of an integration vector containing a

05 regulated mycobacterial promoter and DNA of interest

As described above, a recombinant plasmid vector, pY6014, was produced by inserting the mycobacterial PyrF gene and flanking mycobacterial DNA sequences into pUC19. In order to insert DNA sequences of interest in a precise  
10 location and particular orientation, several restriction sites on the pY6014 vector as well as the original pUC19 polylinker were removed. The pY6014 vector was digested with KpnI and HindIII, blunt ended and self-ligated to produce plasmid vector pY6016 (See Figure 5).

15 The first third of the M. tuberculosis heat shock protein hsp70 was inserted 106 nucleotides downstream of the PyrF gene in the pY6016 vector. The hsp70 DNA isolated from M. tuberculosis is identical to the M. bovis-BCG hsp70 DNA. Approximately 1.8 kb of hsp70 DNA  
20 was cloned into the HpaI site of the pY6016 vector to produce the pY6017 vector (See Figure 6). In this configuration, the NcoI restriction site overlaps the sequence ATG, which is the start codon, initiating hsp70 protein synthesis. The polymerase chain reaction (PCR)  
25 method was used to produce DNA of interest in an orientation which covers the start codon, thereby driving expression of the DNA of interest from the native start sequence. The hsp70 coding sequence was replaced with DNA of interest encoding a protein or polypeptide of  
30 interest with the result that the hsp70 promoter and ribosome binding site drives the expression of the protein or polypeptide of interest.

The pY6017 vector containing the hsp70 gene was first digested to completion with KpnI or XbaI, then subjected to a NcoI partial XbaI digest. This digest was run on a gel to purify the linearized vector which lacks the hsp70 coding sequence. The DNA of interest was synthesized using PCR, and cloned into the NcoI partial-XbaI digested pY6017. Six vectors were produced with DNA of interest in the hsp70 coding sequence. The plasmid vector pY6018 contains the HIV1 gag gene, synthesized as a 1500 bp NcoI-XbaI fragment using PCR; pY6019 contains the HIV1 pol gene, synthesized as a 2720 bp NcoI-XbaI fragment using PCR; pY6020 contains the HIV1 env gene, synthesized as a 2570 bp NcoI-HbaI fragment using PCR; pY6021 contains the SIV1 gag gene, synthesized as a 1520 bp blunt-KpnI fragment using PCR; pY6022 contains the SIV1 pol gene, synthesized as a 3125 bp SmaI fragment using PCR; and pY6023 contains the SIV1 env gene, synthesized as a 2750 bp SmaI-XbaI fragment using PCR (See Figures 7-12).

20 Transformation of the marked mycobacterial target strain  
25 with recombinant plasmid vector and DNA of interest

Standard techniques such as electroporation can be used to introduce the integration vector into the marked mycobacterial target strain (i.e., recombinant mycobacteria containing a selectable marker). As diagrammed in Figure 2, marked BCG cells can be transformed with the integration vector, under conditions appropriate for double homologous recombination to occur between plasmid-borne sequences (i.e., the PyrF gene, homologous mycobacterial sequences flanking the PyrF gene and the DNA of interest, hsp70 promoter and DNA of interest) and homologous sequences in the marked target BCG genome.



-23-

The result is integration of the incoming PyrF-hsp70 promoter-DNA of interest combination and deletion of the aph gene in the marked target BCG. Mycobacterial cells containing the PyrF gene, the hsp70 promoter, and DNA of interest can be selected by culturing the electroporated cells on uracil-containing medium. Only those cells in which integration of the PyrF gene occurs survive.

An important advantage of the method of the present invention, which is illustrated in Figure 2, is that integration of the DNA of interest occurs without concomitant integration of plasmid DNA into the genome. That is, except for the DNA of interest, the net effect is that sequences which are not normally present in mycobacteria (i.e., plasmid sequences) are not present in the recombinant mycobacterial cells.

Overview of uses and advantages of integration vectors  
and methods of the present invention

There are numerous uses for and advantages of the integration vector of the present invention. Several of these are described below, as is the use of the integration vector in constructing expression or vaccine vehicles. As a result of the present invention, DNA of interest is integrated into the mycobacteria genome and expression of the DNA is regulated with a mycobacterial promoter. New genetic approaches to understanding questions of disease pathogenesis are now available. Using the integration vector, it should be possible to insertionally mutagenize and mark genes of pathogenic mycobacteria, by homologous recombination, with the aim of identifying specific genetic functions required for virulence and pathogenesis. For example, using this vector and mycobacteria (e.g., *M. smegmatis*, *M.*

bovis-BCG), virulence genes of M. tuberculosis or M. leprae can be identified and diagnostics (diagnostic tests) developed. By specifically deleting or replacing those genes, it may be possible to develop a more  
05 specific and effective attenuated vaccine against tuberculosis than the current M. bovis-BCG vaccine. Alternatively, as specific protective antigens for tuberculosis and leprosy are identified by study of antigens recognized by T cells from resistant  
10 individuals, it will now be possible to introduce and express them in currently existing M. bovis-BCG vaccines.

Overview of uses and advantages of recombinant  
mycobacteria

The method of the present invention is useful to  
15 construct a genetically recombinant mycobacterial vehicle for the expression of the protein(s) or polypeptide(s) encoded by DNA of interest incorporated into the mycobacterium. Such genetically recombinant mycobacteria have many uses.

20 Vehicles of the present invention can be used, for example, as vaccines to induce immunity against the pathogenic antigen encoded by the DNA of interest. A pathogen is any virus, microorganism, or other organism or substance (e. g., toxins) which causes disease. A  
25 vaccine vehicle useful for immunizing against leprosy can be made. Because of the extraordinary adjuvant activity of mycobacteria, such as BCG, such a vaccine would be effective in producing cell-mediated immunity, particularly of a long-term or enduring nature. Genes encoding  
30 protein antigens of the leprosy parasite M. leprae have been isolated by Young and are described in detail in co-pending U. S. Patent Application Serial No. 892,095,

-25-

filed July 31, 1986, the teachings of which are incorporated herein by reference. In particular, genes encoding five immunogenic protein antigens (i. e., antigens of molecular weight 65kD, 36kD, 28kD, 18kD and 12kD) have been isolated. In addition, 6 different epitopes encoded by the gene for the 65kD antigen have been defined. At least one of these epitopes has been shown to be unique to M. leprae; the other epitopes have been shown to be shared with the 65kD proteins of other mycobacteria.

Through use of the integration vector of the present invention, it is possible to integrate into the BCG genome one or more of the genes encoding M. leprae protein antigens, using methods described above and in the following examples. The genes encoding the HIV1 and the SIV1 gag, pol, and env proteins have been cloned into plasmid vectors, to be introduced into marked target M. bovis-BCG cells using the techniques described above. In this way, it is possible to construct a vaccine which is close to ideal, in that it contains one or more protective antigens of HIV1 or SIV1, does not have tolerogenic determinants and has an excellent adjuvant for inducing cell-mediated immunity.

In a similar fashion, it is possible to construct a vaccine, using an integration vector and the method of the present invention, to provide specific protection against tuberculosis. Genes encoding immunogenic protein antigens of the tubercle bacillus M. tuberculosis have been isolated and are described in co-pending U. S. patent application Serial No. 07/010,007, entitled "Genes Encoding Protein Antigens of Mycobacterium Tuberculosis and Uses Therefor", by Robert N. Husson and Richard A. Young, filed February 2, 1987 (now abandoned), and in the

continuation-in-part application, Serial No. 07/154.331, (filed by the Express Mail procedure February 10, 1988), entitled "Genes Encoding Protein Antigens of Mycobacterium Tuberculosis and Uses Therefor", by Robert N. Husson, Richard A. Young and Thomas M. Shinnick, the teachings of which are incorporated herein by reference.

In this case, a gene encoding an immunogenic protein antigen of M. tuberculosis is integrated into a marked target BCG by means of an integration vector, as described above. It is also possible to integrate more than one M. tuberculosis gene, each encoding a protein antigen, into the BCG genome. For example, a gene encoding immunogenic M. tuberculosis antigens of molecular weight 12kD, 14kD, 19kD, 65kD and 71kD, or a combination of two or more of these genes under the control of regulated mycobacterial promoter region, such as a heat shock protein promoter region or stress protein promoter region, can be inserted into an integration vector, stably integrated into the genomic DNA of marked target BCG and expressed. The result is a vaccine which is specific for immunization against tuberculosis and which induces long-lived immunity against the bacillus.

Vaccine vehicles which express a protein antigen or antigens from malaria sporozoites, malaria merozoites, diphtheria toxoid, tetanus toxoid, Leishmania, Salmonella, Mycobacterium africanum, Mycobacterium intracellulare, Mycobacterium avium, treponema, pertussis, herpes virus, measles virus, mumps, Shigella, Neisseria, Borrelia, rabies, polio virus, HIV-1, Simian immunodeficiency virus, snake venom, insect venom or vibrio cholera can also be produced in the same manner.

-27-

It is also possible, using the method of the present invention, to construct a multipurpose or multifunctional vaccine (i.e., a single vaccine vehicle which contains and expresses DNA of interest which includes more than one gene, each gene encoding a protein antigen for a different pathogen or toxin). For example, it is possible to integrate into the BCG genome, using the integration vector described, a gene encoding a protein antigen for M. leprae, a gene encoding a protein antigen for M. tuberculosis, a gene encoding a protein antigen for Leishmania, and a gene encoding a protein antigen for malaria. Administration of this multi-valent vaccine would result in stimulation of an immune response to each antigen and provide long-term protection against leprosy, tuberculosis, leishmaniasis, and malaria.

The recombinant mycobacteria can also be used as an anti-fertility "vaccine" vehicle. For example, mycobacteria containing DNA encoding proteins such as human gonadotropic hormone (HGH) fragments, can be used as an anti-fertility vaccine and administered as a birth control agent. Vaccine vehicles of the present invention can be used to treat human cancers, such as bladder cancers or melanomas (e.g., by expressing growth inhibitors or cytocidal products). In this context, recombinant mycobacteria which contain and express interferon  $\alpha$ ,  $\beta$  and/or  $\gamma$ , one or more interleukin (interleukins 1-7) and/or TNF  $\alpha$  or  $\beta$  are particularly useful. In another application, recombinant mycobacteria can be used to express stress proteins, either for the purpose of eliciting a protective immune response (e.g., against subsequent or long-term infection) or for the purpose of inducing tolerance in an autoimmune disease (e.g., rheumatoid arthritis). Stress proteins, such as

those described in co-pending U.S. patent application Serial No. 207,298, entitled Stress Proteins and Uses Therefore, by Richard A. Young and Douglas Young, filed June 15, 1988, can be used in this purpose. Because of  
05 their large genomes (e.g., the BCG genome is about  $3 \times 10^6$  bp long), mycobacteria can accommodate large amounts of DNA of interest and, thus, can serve as multi-purpose vehicles.

Recombinant mycobacteria of the present invention  
10 can be used to produce polypeptide(s) of interest, such as those involved in steroid synthesis. In this case, all or a portion of such a gene is integrated into the mycobacterial host, in which expression is regulated as described above. Thus, the recombinant mycobacteria  
15 provide a valuable means of producing such proteins that could synthesize steroids.

In any of the uses of the recombinant mycobacteria to express a protein or polypeptide, it is possible to include in the integration vector DNA encoding a signal  
20 sequence and, thus, provide a means by which the expressed protein or polypeptide is made in the cytoplasm and then secreted at the cell walls. For example, the signal sequence from  $\alpha$  antigen, which is secreted in mycobacteria, could be used. Alternatively, the signal  
25 sequence for  $\beta$ -galactosidase, agarase or  $\alpha$  amylase could be used.

The present invention will now be illustrated by the following examples, which are not to be considered limiting in any way.

-29-

Example 1      Construction of a recombinant plasmid for  
                 introduction of the Kan gene into M.  
                 bovis-BCG and of a marked target BCG

                 The DH5 $\alpha$  bacterial strain was used to prepare a  
05 recombinant plasmid. (Bethesda Research Lab, Maryland).  
                 M. bovis-BCG (Moreau) genomic DNA was prepared by  
                 standard procedures.

                 The starting vector pY6014 was produced by inserting  
                 a SacI partial-EcoRI 4510 bp fragment cloned from pY6013  
10 into pUC19 DNA digested with SacI and EcoRI. (See  
                 Figure 3) The pY6011 plasmid was obtained by inserting a  
                 5610 bp EcoRI fragment from Y3330 into EcoRI digested  
                 pGEM-7Zf<sup>+</sup> (Promega Biotech). Y3330 is a  $\lambda$ gt11 clone from  
                 a  $\lambda$ gy11 library of BCG DNA, obtained by screening the  
15 library with a fragment of the BCG PryF gene. The pY6014  
                 contains the mycobacterial PyrF gene and 1.4-1.6 kb of  
                 flanking mycobacterial DNA on either side of the PyrF  
                 gene.

                 The aph gene which encodes kanamycin resistance was  
20 obtained from pY6005 and inserted in the pY6014  
                 recombinant plasmid to replace the PyrF coding sequences,  
                 using standard techniques. (pY6005; (Kan<sup>R</sup>) kanamycin  
                 resistance cartridge derived from Tn903 was inserted in  
                 pUC4 SacI, a derivative of pUC4 with pUC1 replacing the  
25 SacI site in the polylinker). The aph gene was cloned  
                 into HincII 6.1 kb fragment from pY6014, as a BamHI  
                 filled in fragment from pY6005. This produced the  
                 recombinant plasmid pY6015 which was used to produce the  
                 marked target BCG strain. (See Figure 4)

-30-

Isolation of marked target BCG containing the Kan gene

As diagrammed in Figure 1, a marked target BCG strain was produced by transforming BCG with the recombinant plasmid pY6015 described above. This  
05 procedure was performed as follows: BCG cells were grown in suspension culture at optical density (OD) 0.5, concentrated 50 fold in electroporation buffer PSB (phosphate sucrose buffer) and transferred to a curvette. After addition of 10 µg of plasmid DNA, one electrical  
10 pulse of 6,250 V/cm and 25 µF was given and the cells were resuspended in 10 ml medium, grown at 37°C for 2 hours and plated.

Marked target BCG were isolated by plating the transformation on Middlebrook 7H10 agar plates containing  
15 20 µg/ml kanamycin and 1 mg/ml FOA.

Example 2 Construction of an integration vector for  
introduction of DNA of interest into marked  
target M. bovis-BCG-----

An integration vector capable of transforming the  
20 marked target BCG (produced in Example 1) was engineered which contained the mycobacterial PyrF gene, DNA sequences homologous to sequences flanking mycobacterial PyrF sequences necessary for homologous recombination, the mycobacterial hsp70 promoter and DNA of interest  
25 (i.e., the HIV1 or SIV1 gag, pol or env gene). This procedure is described below and diagrammed in Figure 2.

The recombinant vector pY6014, described in Example 1, which contains the mycobacterial PyrF gene and flanking DNA sequences was digested with KpnI and  
30 HindIII, blunt ended and self-ligated to produce the vector pY6016. (See Figure 5). This procedure was



-31-

performed according to standards procedures. This results in the removal of several restriction sites present in pY6014 located in the original polylinker DNA sequence.

05 The hsp70 M. tuberculosis EcoRI-HindIII 1.8 kb filled in fragment was obtained from Y3334 (an M13 mp18 phage containing the EcoRI - Kpm 1.8 Kb fragment from  $\lambda$ gt11 clone Y3111 which was obtained by screening a  $\lambda$ gt11 library of M. tuberculosis DNA with antibodies against  
10 the 70 kDa antigen), and cloned into the HpaI site of pY6016 (106 bp downstream of PyrF) using standard procedures.

As shown in Figure 6, this results in the recombinant plasmid pY6017, which contains the hsp70  
15 promoter and partial coding sequence as well as mycobacterial (BCG) flanking sequences necessary for double homologous recombination with the marked target BCG produced in Example 1. Using this vector (pY6017), 6 integration vectors were produced having DNA encoding the  
20 HIV1 gag, pol or env gene or the SIV1 gag, pol or env ene. Each of these vectors was produced by the following procedure in which the hsp70 coding region was replaced with the DNA of interest.

#### Insertion of DNA of interest in pY6017

25 The NcoI site in the hsp70 insert is at the ATG of hsp70 gene. DNA encoding a gene of interest was cloned into this vector (pY6017) using a polymerase chain reaction (PCR) on the gene of interest as template. The two oligonucleotides used in the reaction contain at the  
30 two ends two convenient restriction sites (in this case, NcoI for the oligonucleotides at the 5' end of the gene,

and one of the sites of the polylinker for the oligonucleotides at the 3' end of the gene). The PCR product was digested with the two enzymes chosen for the oligonucleotides and gel purified. The vector was cut to completion with one of the enzymes of the polylinker (KpnI, XbaI) and to partial digestion with NcoI. If NcoI was present in the gene of interest, the NcoI site was blunted prior to digestion with the KpnI or XbaI enzyme. The PCR product was filled in at the 5' end, and cut with the 3' end enzyme.

The following recombinant plasmid vectors were produced:

- 15 pY6018: the HIV1 gag gene was synthesized as a 1500 bp NcoI-XbaI fragment using PCR and cloned into NcoI partial-XbaI digested pY6017 (See Figure 7)
- 20 pY6019: the HIV1 pol gene was synthesized as a 2720 bp NcoI-XbaI fragment using PCR and was inserted into NcoI partial-XbaI digested pY6017 (See Figure 8)
- pY6020: the HIV1 env gene was synthesized as a 2570 bp NcoI-XbaI fragment using PCR and was inserted into NcoI partial-XbaI digested pY6017 (See Figure 9)
- 25 pY6021: the SIV1 gag gene was synthesized as a 1520 bp blunt- KpnI fragment using PCR and was inserted into NcoI partial-filled in KpnI digested pY6017 (See Figure 10)
- 30 pY6022: the SIV1 pol gene was synthesized as a 3125 bp SmaI fragment using PCR and was inserted into NcoI partial-XbaI digested and filled in pY6017 (See Figure 11)

-33-

pY6023: the SIV1 env gene was synthesized as a 2750 bp  
SmaI-XbaI fragment using PCR and was inserted  
into NcoI partial filled in and XbaI digested  
pY6017 (See Figure 12)

CLAIMS

1. A recombinant mycobacterium comprising DNA of interest and a regulated promoter region, said DNA of interest ~~and said promoter region~~ stably integrated into the genomic DNA of said mycobacterium in such a manner that expression of said DNA of interest is under the control of the regulated promoter region.
2. A recombinant mycobacterium of Claim 1 which is recombinant BCG; the DNA of interest encodes a protein or polypeptide of interest selected from the group consisting of: antigens, enzymes, lymphokines, immunopotentiators and reporter molecules; and the regulated promoter region is a mycobacterial promoter region.
3. A recombinant mycobacterium of Claim 1, which is selected from the group consisting of:
  - a. Mycobacterium smegmatis;
  - b. Mycobacterium bovis-BCG;
  - c. Mycobacterium avium;
  - d. Mycobacterium phlei;
  - e. Mycobacterium fortuitum;
  - f. Mycobacterium lufu;
  - g. Mycobacterium paratuberculosis;
  - h. Mycobacterium habana;
  - i. Mycobacterium scrofulaceum;
  - j. Mycobacterium intracellulare;
  - k. Mycobacterium tuberculosis; and
  - l. any genetic variants thereof.

-35-

4. Recombinant BCG comprising DNA of interest and a regulated mycobacterial promoter region.
5. A recombinant mycobacterium of Claim 2 wherein the regulated mycobacterial promoter is a heat shock protein promoter region.
6. A vaccine comprising the recombinant mycobacterium of Claim 1 and an appropriate carrier.
7. An integration vector comprising:
  - a) DNA sequences homologous to DNA sequences in the mycobacterial genome;
  - b) DNA encoding a regulated mycobacterial promoter region;
  - c) DNA encoding a selectable marker of mycobacterial origin; and
  - d) DNA encoding at least one protein or at least one polypeptide to be expressed in mycobacteria.
8. An integration vector of Claim 7 wherein the regulated mycobacterial promoter region is a heat shock protein promoter region.
9. An integration vector of Claim 8 wherein the regulated mycobacterial promoter region is the hsp70 promoter region.
10. An integration vector of Claim 7 wherein the protein or polypeptide of interest is selected from the group consisting of: antigens, enzymes, lymphokines, immunopotentiators and reporter molecules.

11. An integration vector of Claim 10 wherein the protein or polypeptide is selected from the group consisting of: HIV gag, HIV pol, HIV env, SIV gag, SIV pol and SIV env.
- 05 12. A method of immunizing a mammalian host against one or more pathogens, comprising administering to said host a recombinant mycobacterium, said recombinant mycobacterium having stably integrated into its genome a) DNA of interest encoding at least one protein antigen for each of said pathogens, and b) DNA encoding a regulated mycobacterial promoter region in such a manner that expression of said DNA of interest is under the control of the regulated mycobacterial promoter region.
- 10
- 15 13. A method of Claim 12 wherein the recombinant mycobacterium is selected from the group consisting of:
- a. Mycobacterium smegmatis;
  - b. Mycobacterium bovis-BCG;
  - 20 c. Mycobacterium avium;
  - d. Mycobacterium phlei;
  - e. Mycobacterium fortuitum;
  - f. Mycobacterium lufu;
  - g. Mycobacterium paratuberculosis;
  - 25 h. Mycobacterium habana;
  - i. Mycobacterium scrofulaceum;
  - j. Mycobacterium intracellulare;
  - k. Mycobacterium tuberculosis; and
  - k. any genetic variants thereof.

- 37 -

14. A method of Claim 13 wherein DNA of interest encodes at least one protein antigen selected from the group consisting of:

a) antigens selected from the group consisting of:

- 05 1. Mycobacterium leprae antigens;
- 2. Mycobacterium tuberculosis antigens;
- 3. malaria sporozoites;
- 4. malaria merozoites;
- 5. diphtheria toxoid;
- 10 6. tetanus toxoids;
- 7. Leishmania antigens;
- 8. Salmonella antigens;
- 9. Mycobacterium africanum antigens;
- 10. Mycobacterium intracellulare antigens;
- 15 11. Mycobacterium avium antigens;
- 12. Treponema antigens;
- 13. Pertussis antigens;
- 14. Herpes virus antigens;
- 15. Measles virus antigens;
- 20 16. Mumps virus antigens;
- 17. Shigella antigens;
- 18. Neisseria antigens;
- 19. Borrelia antigens;
- 20. rabies antigens;
- 25 21. polio virus antigens;
- 22. Human immunodeficiency virus antigens;
- 23. snake venom antigens;
- 24. insect venom antigens; and
- 25. vibrio cholera
- 30 b) steroid enzymes;
- c) interleukins 1 through 7;
- d) tumor necrosis factor  $\alpha$  and  $\beta$ ;
- e) interferon  $\alpha$ ,  $\beta$  and  $\gamma$ ; and

- f) reporter molecules selected from the group consisting of luciferase;  $\beta$ -galactosidase;  $\beta$ -glucuronidase and catechol dehydrogenase.

05 15. A method of making a vaccine for immunization of a mammalian host against one or more pathogens, comprising:

- a) transforming mycobacteria with a recombinant plasmid comprising:

- 10 1) DNA encoding a selectable marker; and  
2) DNA sequences homologous to DNA sequences in the mycobacterial genome;

under conditions appropriate for double homologous recombination to occur between plasmid-borne mycobacterial sequences  
15 homologous to sequences in the mycobacterial genome, thereby producing target mycobacteria cells; and

- b) transforming the target mycobacterial cells of (a) with an integration vector comprising:

- 20 1) DNA of interest encoding at least one protein antigen for each of said pathogens;  
2) DNA encoding a selectable marker of mycobacterial origin;  
25 3) DNA encoding a regulated mycobacterial promoter region capable of regulating the expression of said DNA of interest; and  
4) DNA sequences homologous to DNA sequences in the target mycobacterial genome,  
30 under conditions appropriate for double homologous recombination to occur between plasmid-borne mycobacterial sequences



-39-

homologous to sequences in the target mycobacterial genome, thereby producing a recombinant mycobacterium.

16. A method of Claim 15 wherein the DNA of interest  
05 encodes at least one protein antigen selected from  
the group consisting of:
- a) antigens selected from the group consisting of:
1. Mycobacterium leprae antigens;
  2. Mycobacterium tuberculosis antigens;
  - 10 3. malaria sporozoites;
  4. malaria merozoites;
  5. diphtheria toxoid;
  6. tetanus toxoids;
  7. Leishmania antigens;
  - 15 8. Salmonella antigens;
  9. Mycobacterium africanum antigens;
  10. Mycobacterium intracellulare antigens;
  11. Mycobacterium avium antigens;
  12. Treponema antigens;
  - 20 13. Pertussis antigens;
  14. Herpes virus antigens;
  15. Measles virus antigens;
  16. Mumps virus antigens;
  17. Shigella antigens;
  - 25 18. Neisseria antigens;
  19. Borrelia antigens;
  20. rabies antigens;
  21. polio virus antigens;
  22. Human immunodeficiency virus antigens; HIV
  - 30 23. snake venom antigens;
  24. insect venom antigens; and
  25. vibrio cholera

- b) steroid enzymes;
- c) interleukins 1 through 7;
- d) tumor necrosis factor  $\alpha$  and  $\beta$ ;
- e) interferon  $\alpha$ ,  $\beta$  and  $\gamma$ ; and
- f) reporter molecules selected from the group consisting of luciferase;  $\beta$ -galactosidase;  $\beta$  glucuronidase and catechol dehydrogenase.

17. A method of Claim 15 wherein the regulated mycobacterial promoter region is a heat shock protein promoter region.

18. A method of integrating into a mycobacterial genome DNA encoding a protein or a polypeptide to be expressed in the mycobacterium, comprising the steps of:

- a) transforming mycobacteria with a recombinant plasmid comprising:
  - 1) DNA encoding a selectable marker; and
  - 2) DNA sequences homologous to sequences in mycobacterial genomic DNA,under conditions appropriate for double homologous recombination to occur between plasmid-borne mycobacterial sequences homologous to sequences in the mycobacterial genome, thereby producing a target mycobacterial cells;
- b) selecting target mycobacterial cells produced in (a) in which double homologous recombination has occurred;
- c) transforming the target mycobacterial cells of (b) with an integration vector, comprising:

-41-

- 1) DNA sequences homologous to DNA sequences in the target mycobacterial genome;
  - 2) DNA encoding a selectable marker of mycobacterial origin;
  - 05 3) DNA encoding a regulated mycobacterial promoter region; and
  - 4) DNA encoding the protein or polypeptide to be expressed, under conditions appropriate for double homologous recombination to occur between  
10 plasmid-borne mycobacterial sequences homologous to sequences in the target mycobacterial genome, thereby producing recombinant mycobacteria containing DNA of  
15 interest; and
  - d) selecting recombinant mycobacterial cells produced in (c) in which double homologous recombination has occurred.
19. A method of Claim 18 wherein the protein or  
20 polypeptide of interest is selected from the group consisting of: antigens, enzymes, lymphokines, immunopotentiators and reporter molecules.
20. A method of Claim 18 wherein the regulated  
25 mycobacterial promoter is a heat shock protein promoter region.
21. A method of immunizing a mammalian host against one or more pathogens, comprising administering to said host a recombinant mycobacterium, said recombinant mycobacterium having stably integrated into its  
30 genome a) DNA encoding at least one protein antigen

for each of said pathogens, and b) DNA encoding a regulated mycobacterial promoter region in such a manner that expression of the DNA of interest is under the control of the regulated mycobacterial promoter region.

05

22. A recombinant mycobacterium of Claim 21, wherein the DNA of interest encodes at least one protein antigen selected from the group consisting of:

a) antigens selected from the group consisting of:

10

1. Mycobacterium leprae antigens;
2. Mycobacterium tuberculosis antigens;

3. malaria sporozoites;

4. malaria merozoites;

5. diphtheria toxoid;

15

6. tetanus toxoids;

7. Leishmania antigens;

8. Salmonella antigens;

9. Mycobacterium africanum antigens;

10. Mycobacterium intracellulare antigens;

20

11. Mycobacterium avium antigens;

12. Treponema antigens;

13. Pertussis antigens;

14. Herpes virus antigens;

15. Measles virus antigens;

25

16. Mumps virus antigens;

17. Shigella antigens;

18. Neisseria antigens;

19. Borrelia antigens;

20. rabies antigens;

30

21. polio virus antigens;

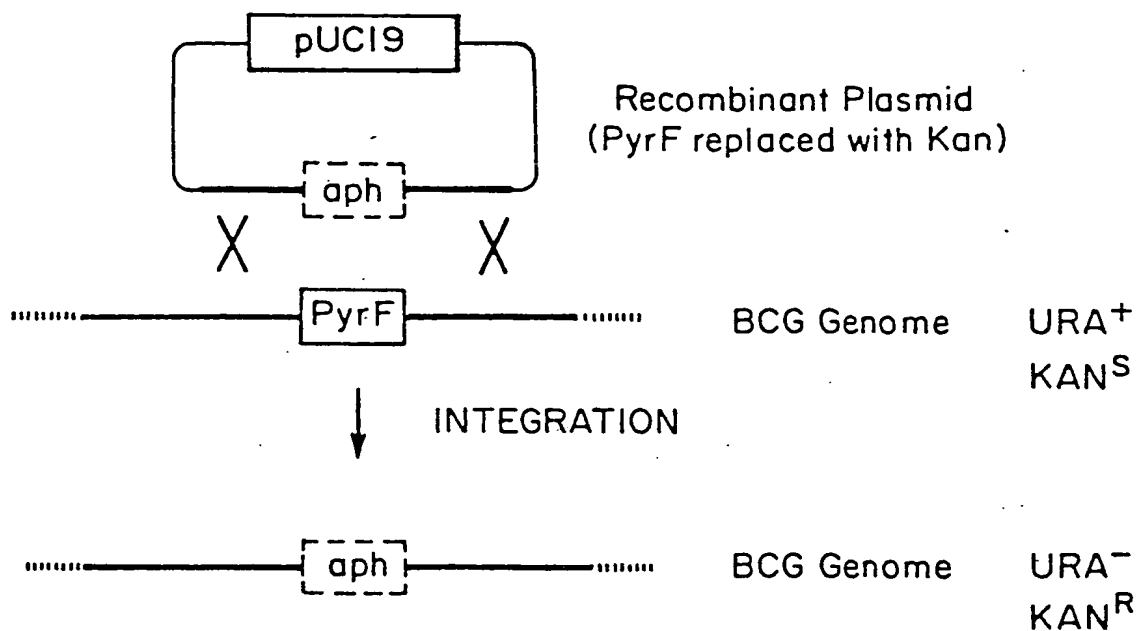
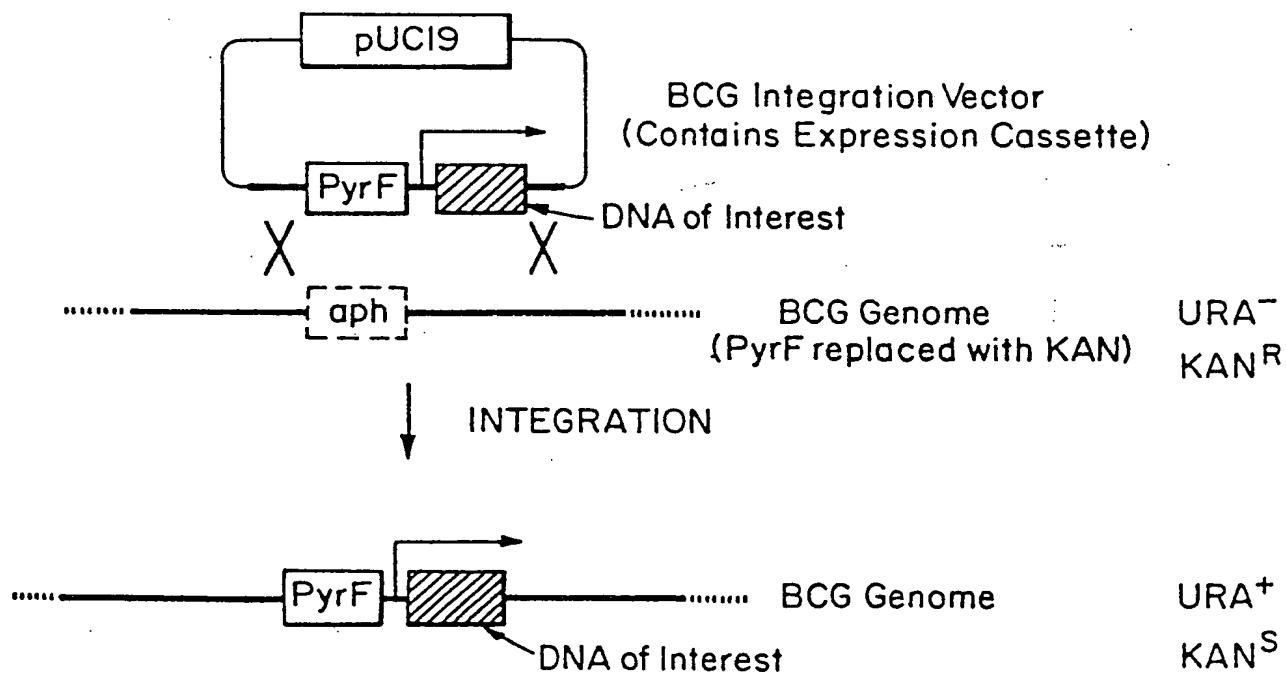
22. Human immunodeficiency virus antigens;

23. Simian immunodeficiency virus antigens;

-43-

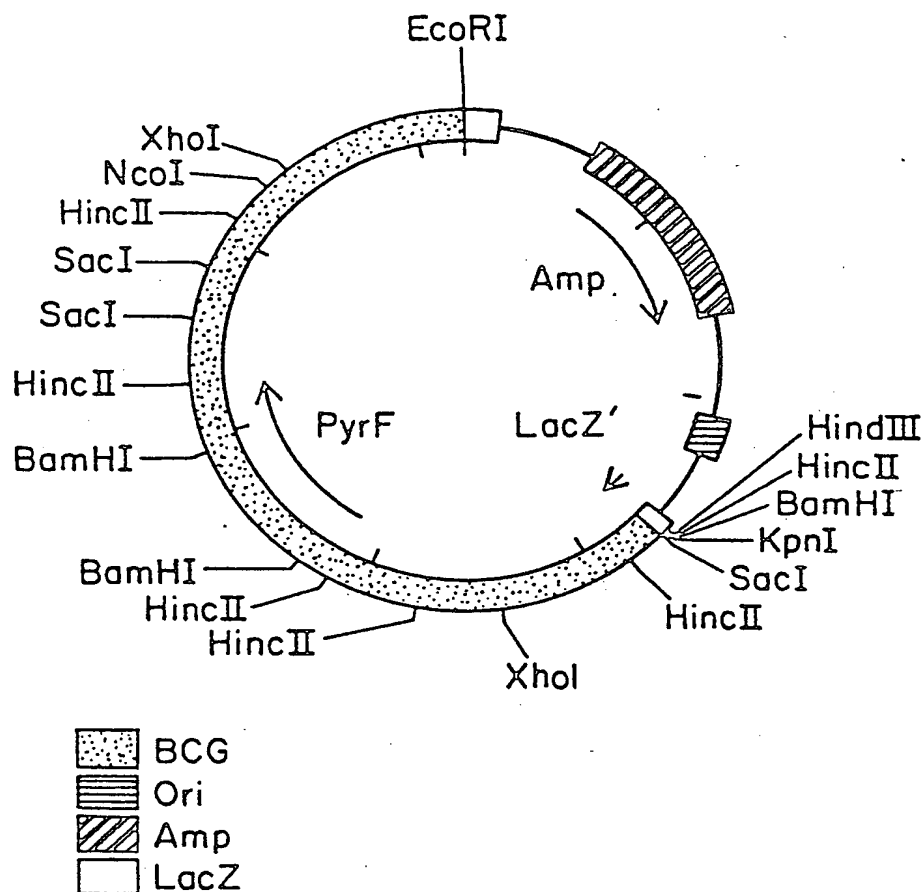
- 24. snake venom antigens;
  - 25. insect venom antigens; and
  - 26. vibrio cholera
- 05      b) steroid enzymes;
- c) interleukins 1 through 7;
- d) tumor necrosis factor  $\alpha$  and  $\beta$ ;
- e) interferon  $\alpha$ ,  $\beta$  and  $\gamma$ ; and
- f) reporter molecules selected from the group
- 10      consisting of luciferase;  $\beta$ -galactosidase;  $\beta$
- glucuronidase and catechol dehydrogenase.

1/11

*Fig. 1**Fig. 2*

SUBSTITUTE SHEET

2/11  
pY6014 (pBAA2)



RESTRICTION SITES

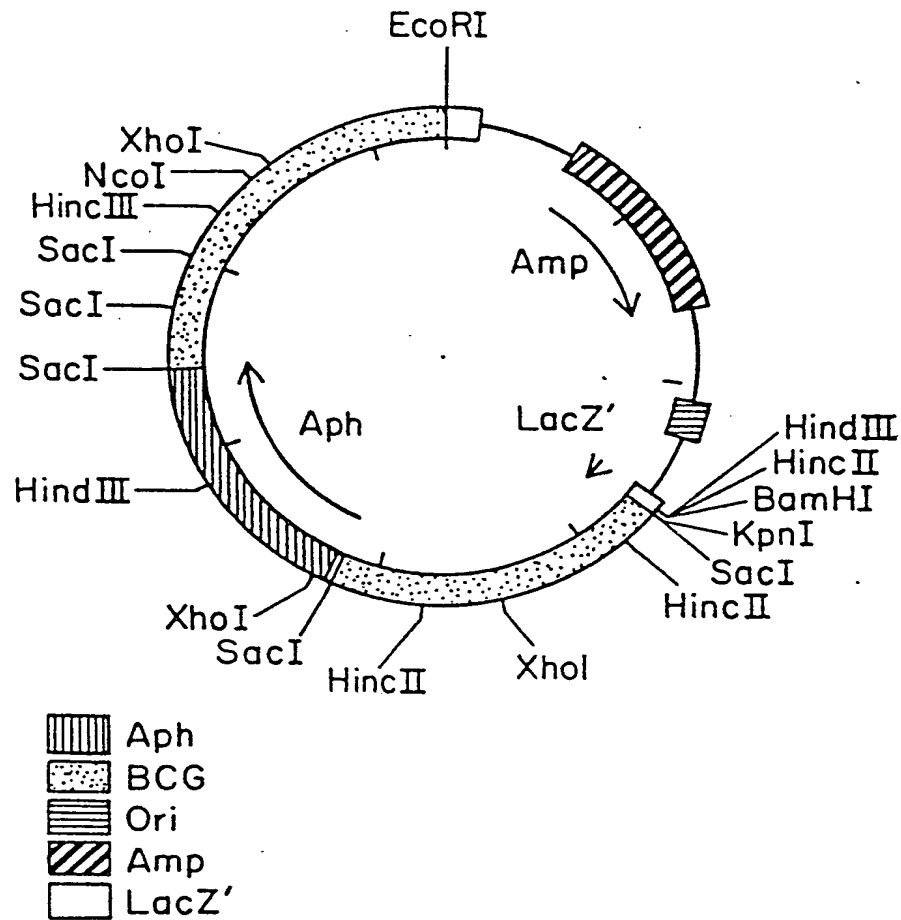
|         |      |      |      |      |
|---------|------|------|------|------|
| BamHI   | 2666 | 4320 | 4960 |      |
| EcoRI   | 1    |      |      |      |
| HincII  | 2654 | 2850 | 3780 | 4180 |
|         | 5290 | 6090 |      |      |
| HindIII | 2638 |      |      |      |
| KpnI    | 2675 |      |      |      |
| NcoI    | 6290 |      |      |      |
| SacI    | 2681 | 5590 | 5840 |      |
| XhoI    | 3430 | 6390 |      |      |

*Fig. 3*

SUBSTITUTE SHEET

3/11

pY6015 (pBAA3)



RESTRICTION SITES

|         |      |      |      |      |
|---------|------|------|------|------|
| BamHI   | 2666 |      |      |      |
| EcoRI   | 1    |      |      |      |
| HindII  | 2654 | 2850 | 3780 | 6247 |
| HincIII | 2638 | 4888 |      |      |
| KpnI    | 2675 |      |      |      |
| NcoI    | 6447 |      |      |      |
| SacI    | 2681 | 4189 | 5438 | 5747 |
|         | 5997 |      |      |      |
| XhoI    | 3430 | 4259 | 6547 |      |

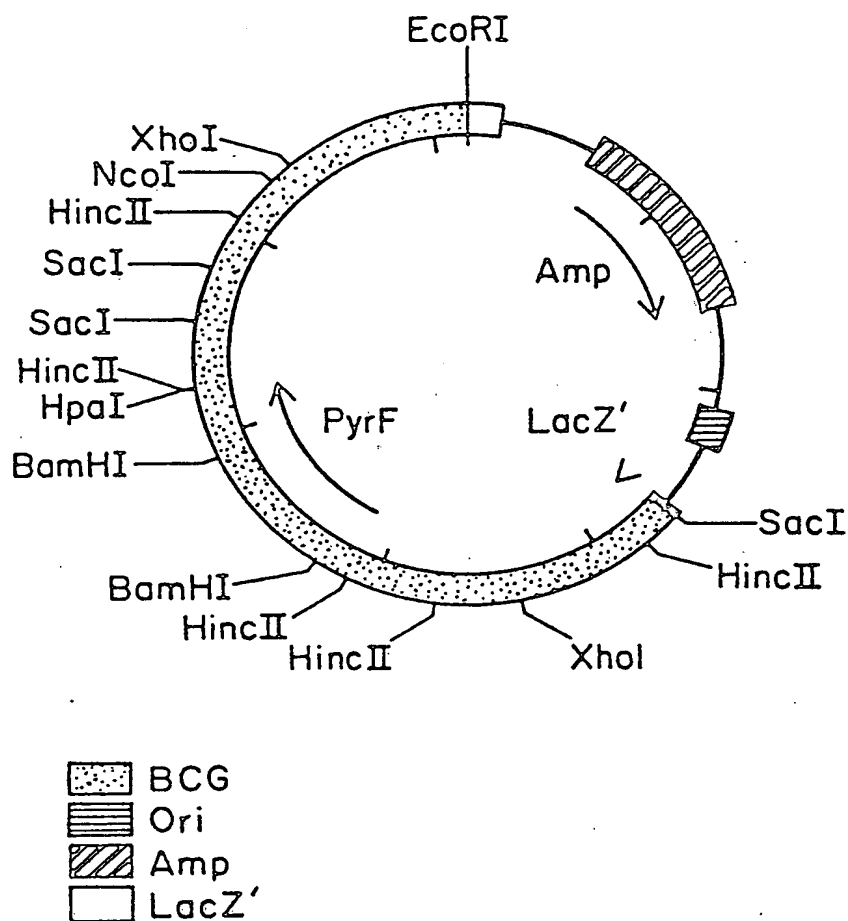
*Fig. 4*

SUBSTITUTE SHEET



4/11

pY6016 (pBAA4)



RESTRICTION SITES

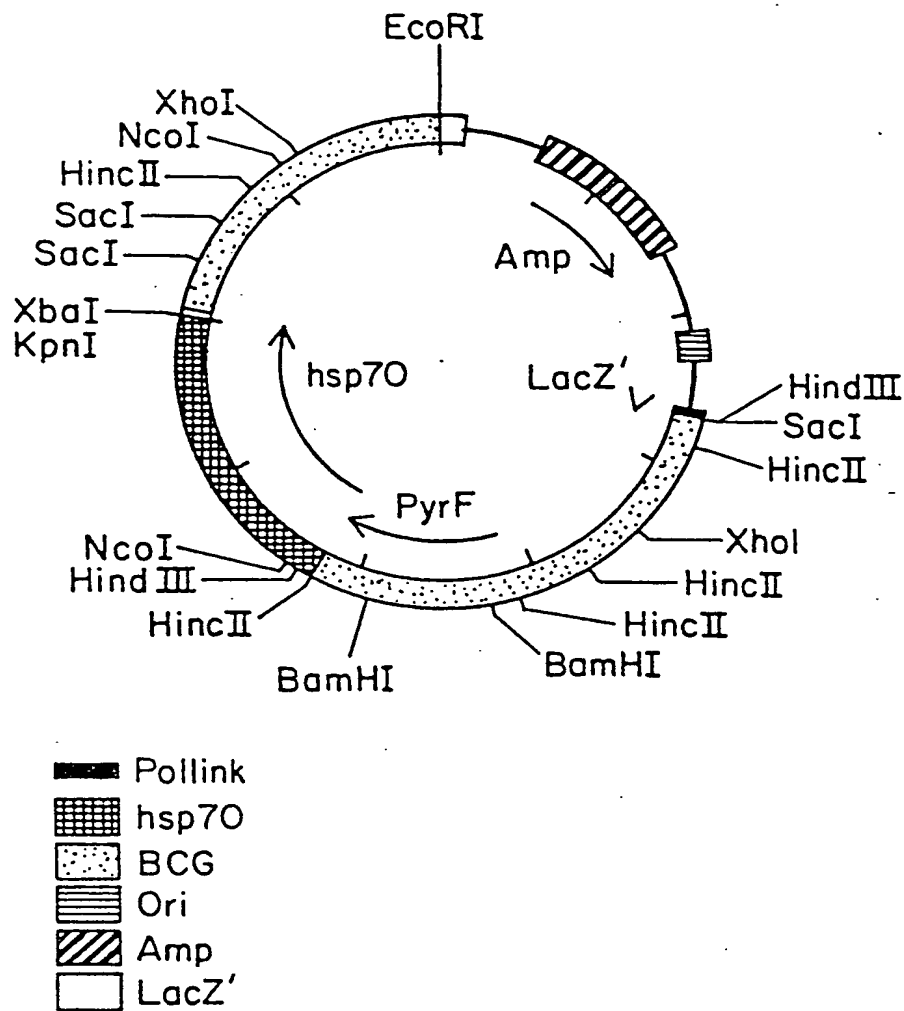
|        |      |      |      |      |
|--------|------|------|------|------|
| BamHI  | 4283 | 4923 |      |      |
| EcoRI  | 1    |      |      |      |
| HincII | 2813 | 3743 | 4143 | 5253 |
|        | 6053 |      |      |      |
| HpaI   | 5253 |      |      |      |
| NcoI   | 6253 |      |      |      |
| SacI   | 2644 | 5553 | 5803 |      |
| XhoI   | 3393 | 6353 |      |      |

*Fig. 5*

SUBSTITUTE SHEET

5/11

pY6017 (pBAA5)



RESTRICTION SITES

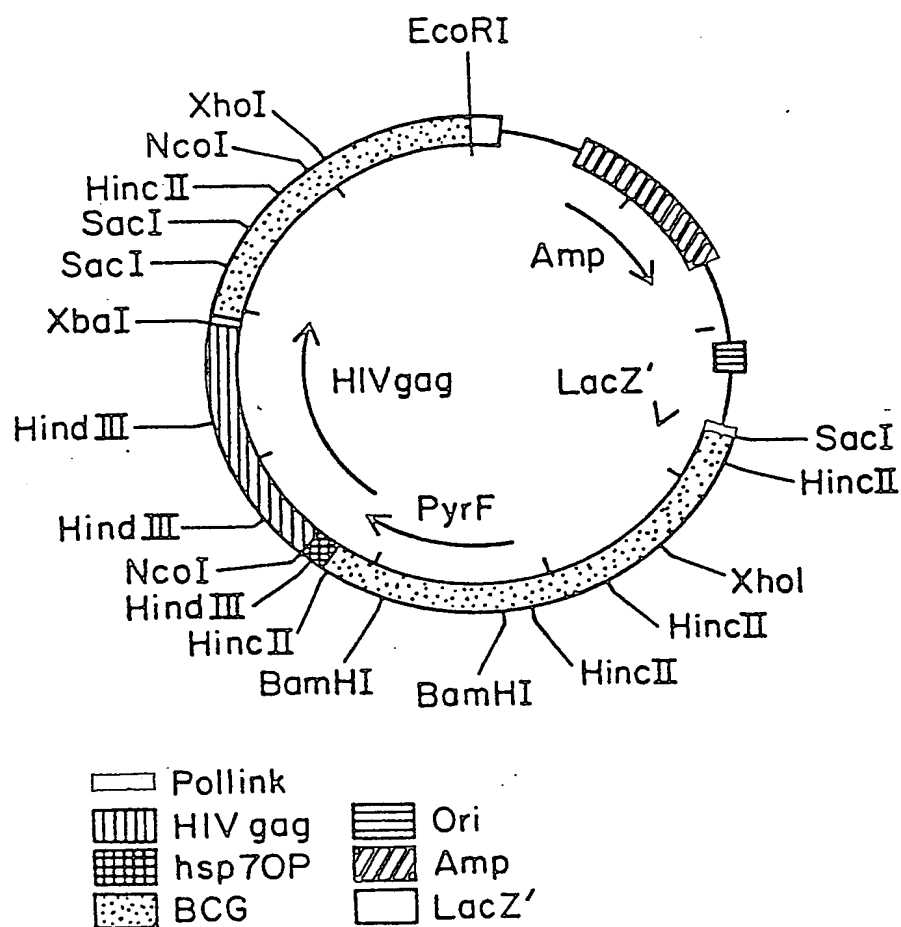
|         |      |      |      |      |
|---------|------|------|------|------|
| BamHI   | 4283 | 4923 |      |      |
| EcoRI   | 1    |      |      |      |
| HincII  | 2813 | 3743 | 4143 | 5253 |
|         | 7853 |      |      |      |
| HindIII | 2638 | 5344 |      |      |
| KpnI    | 7014 |      |      |      |
| NcoI    | 5408 | 8053 |      |      |
| SacI    | 2644 | 7353 | 7603 |      |
| XbaI    | 7025 |      |      |      |
| XhoI    | 3393 | 8153 |      |      |

*Fig. 6*

**SUBSTITUTE SHEET**

6/11

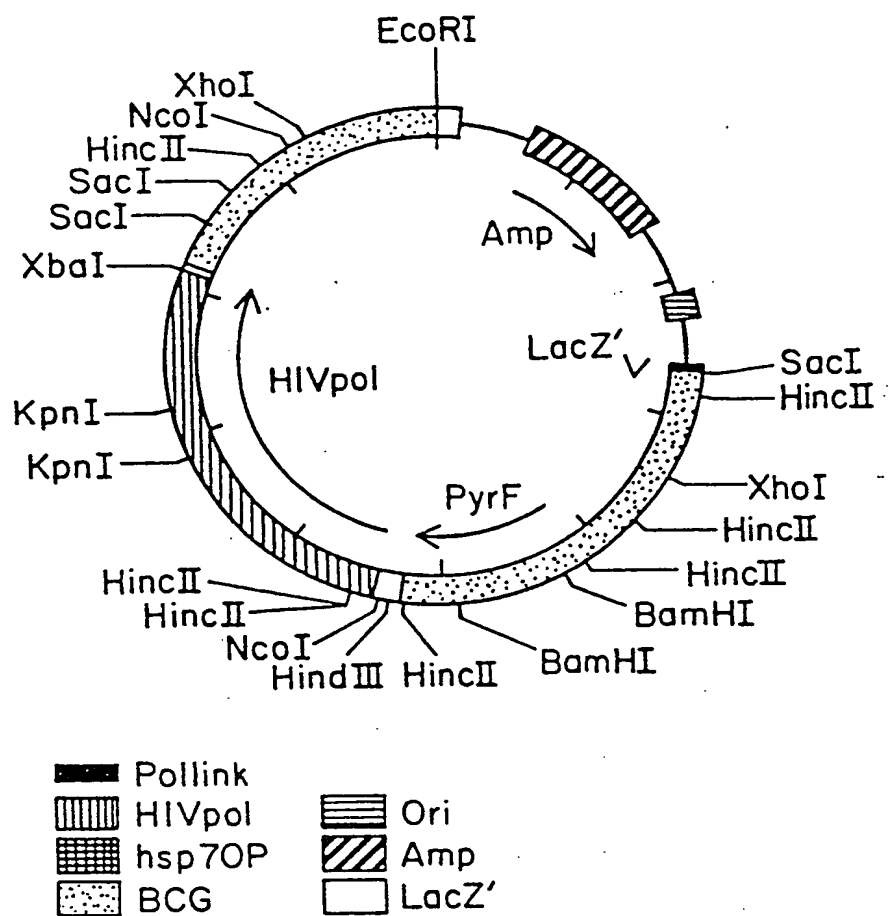
pY6018 (pBAA6)



## RESTRICTION SITES

|         |      |      |      |      |
|---------|------|------|------|------|
| BamHI   | 4283 | 4923 |      |      |
| EcoRI   | 1    |      |      |      |
| HincII  | 2813 | 3743 | 4143 | 5253 |
|         | 7736 |      |      |      |
| HindIII | 5344 | 5698 | 6328 |      |
| NcoI    | 5408 | 7936 |      |      |
| SacI    | 2644 | 7236 | 7486 |      |
| XbaI    | 6908 |      |      |      |
| XhoI    | 3393 | 8036 |      |      |

Fig. 7

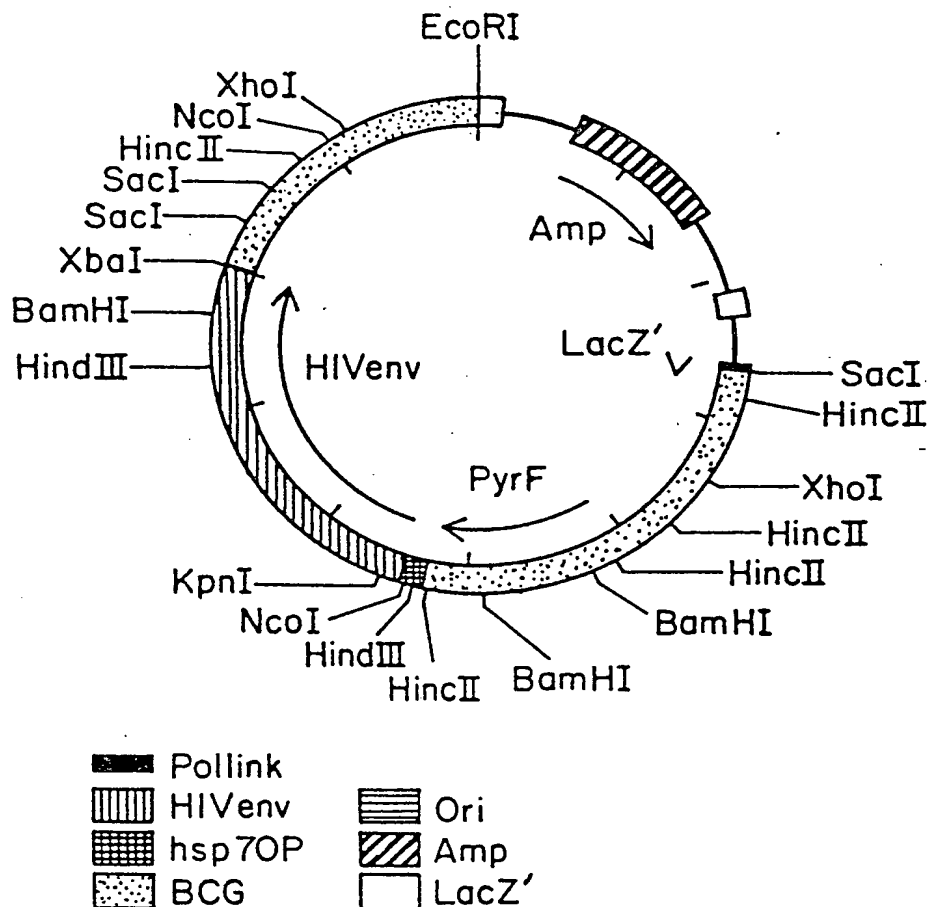
pY6019 (pBAA7)RESTRICTION SITES

|         |      |      |      |      |
|---------|------|------|------|------|
| BamHI   | 4283 | 4923 |      |      |
| EcoRI   | 1    |      |      |      |
| HincII  | 2813 | 3743 | 4143 | 5253 |
|         | 5548 | 5568 | 8956 |      |
| HindIII | 5344 |      |      |      |
| KpnI    | 6888 | 7218 |      |      |
| NcoI    | 5408 | 9156 |      |      |
| SacI    | 2644 | 8456 | 8706 |      |
| XbaI    | 8128 |      |      |      |
| XhoI    | 3393 | 9256 |      |      |

Fig. 8

SUBSTITUTE SHEET

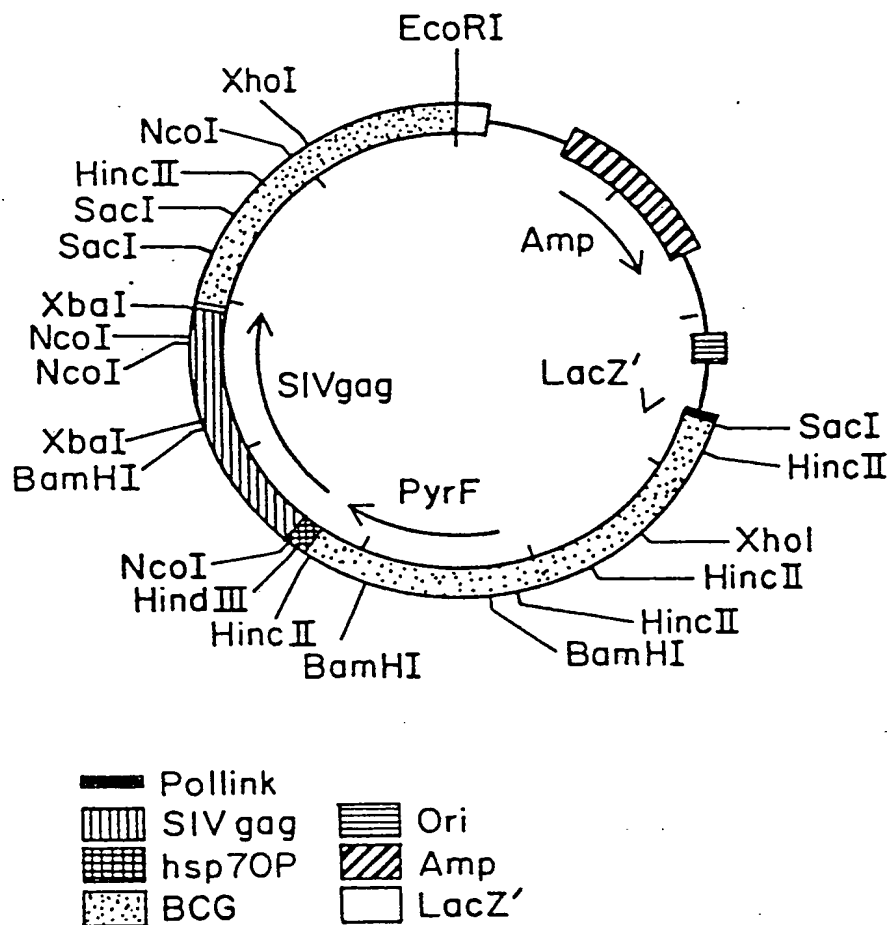
8/11

pY6020 (pBAA8)RESTRICTION SITES

|         |      |      |      |      |
|---------|------|------|------|------|
| BamHI   | 4283 | 4923 | 7663 |      |
| EcoRI   | 1    |      |      |      |
| HincII  | 2813 | 3743 | 4143 | 5253 |
|         | 8806 |      |      |      |
| HindIII | 5344 | 7328 |      |      |
| KpnI    | 5553 |      |      |      |
| NcoI    | 5408 | 9006 |      |      |
| SacI    | 2644 | 8306 | 8556 |      |
| XbaI    | 7978 |      |      |      |
| XhoI    | 3393 | 9106 |      |      |

*Fig. 9*

SUBSTITUTE SHEET

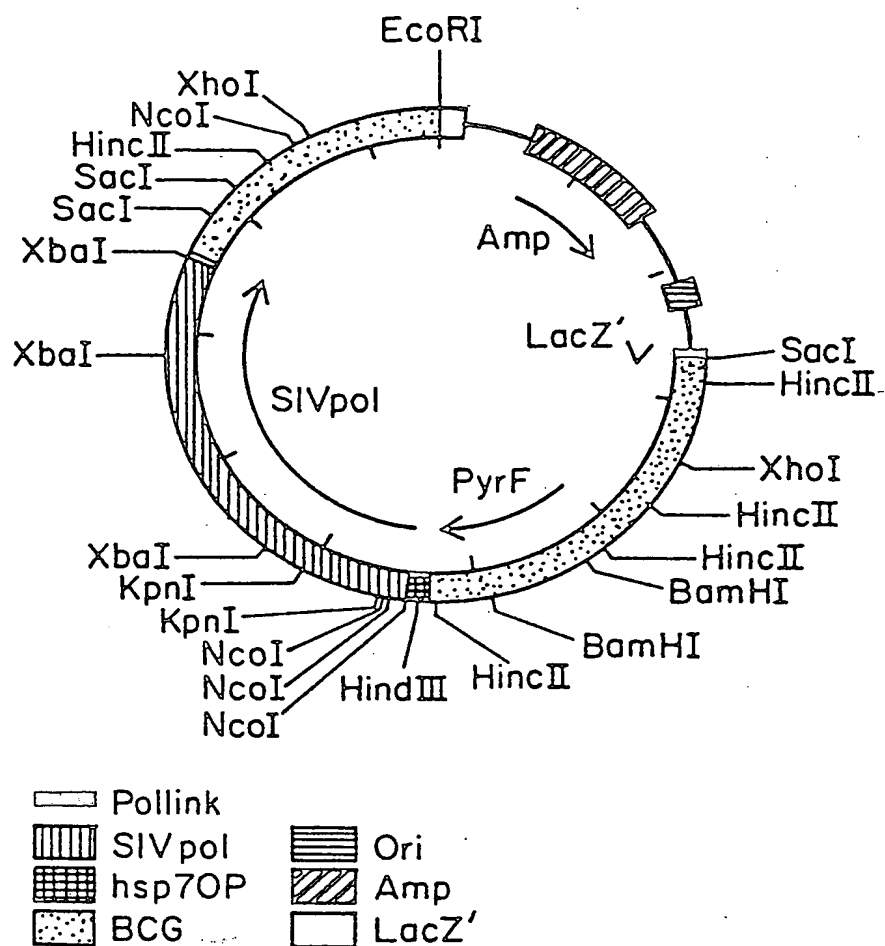
pY6021 (pBAA9)RESTRICTION SITES

|         |      |      |      |      |
|---------|------|------|------|------|
| BamHI   | 4283 | 4923 | 6216 |      |
| EcoRI   | 1    |      |      |      |
| HincII  | 2813 | 3743 | 4143 | 5253 |
|         | 7756 |      |      |      |
| HindIII | 5344 |      |      |      |
| NcoI    | 5408 | 6728 | 6758 | 7956 |
| SacI    | 2644 | 7256 | 7506 |      |
| XbaI    | 6258 | 6928 |      |      |
| XhoI    | 3393 | 8056 |      |      |

*Fig. 10*

SUBSTITUTE SHEET

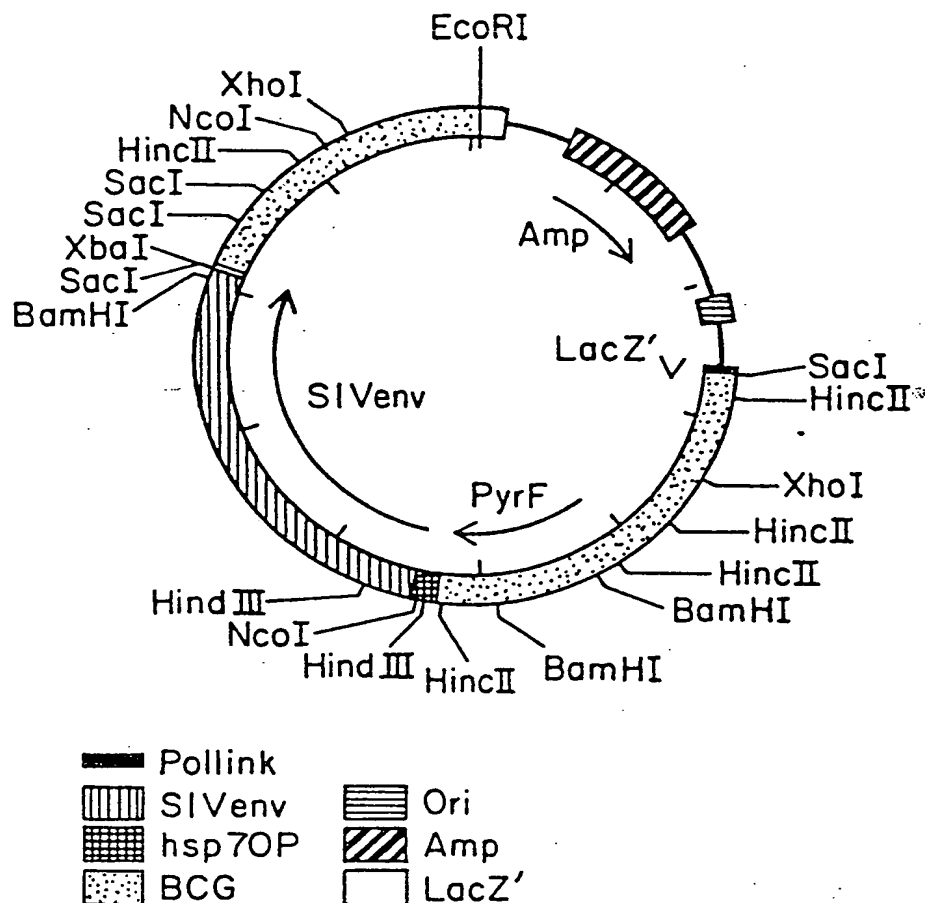
10/11

pY6022 (pBAA10)RESTRICTION SITES

|         |      |      |      |      |
|---------|------|------|------|------|
| BamHI   | 4283 | 4923 |      |      |
| EcoRI   | 1    |      |      |      |
| HincII  | 2813 | 3743 | 4143 | 5253 |
|         | 9361 |      |      |      |
| HindIII | 5344 |      |      |      |
| KpnI    | 5573 | 6073 |      |      |
| NcoI    | 5408 | 5508 | 5538 | 9561 |
| SacI    | 2644 | 8861 | 9111 |      |
| XbaI    | 6343 | 7853 | 8533 |      |
| XhoI    | 3393 | 9661 |      |      |

*Fig. 11*

SUBSTITUTE SHEET

pY6023 (pBAA11)RESTRICTION SITES

|         |      |      |      |      |
|---------|------|------|------|------|
| BamHI   | 4283 | 4923 | 8068 |      |
| EcoRI   | 1    |      |      |      |
| HincII  | 2813 | 3743 | 4143 | 5253 |
|         | 8951 |      |      |      |
| HindIII | 5344 | 5708 |      |      |
| NcoI    | 5408 | 9151 |      |      |
| SacI    | 2644 | 8110 | 8451 | 8701 |
| XbaI    | 8123 |      |      |      |
| XhoI    | 3393 | 9251 |      |      |

Fig. 12

SUBSTITUTE SHEET



# INTERNATIONAL SEARCH REPORT

International Application No **PCT/US 90/03451**

|  |  |  |
|--|--|--|
| <b>I. CLASSIFICATION OF SUBJECT MATTER</b> (if several classification symbols apply, indicate all) <sup>6</sup><br>According to International Patent Classification (IPC) or to both National Classification and IPC<br>IPC <sup>5</sup> : C 12 N 15/74, 15/48, A 61 K 39/04, 39/21, C 12 N 1/21,<br>IPC <sup>5</sup> : //(C 12 N 1/21, C 12 R 1:32)(C 12 N 15/74, C 12 R 1:32)  |  |  |
| <b>II. FIELDS SEARCHED</b><br><div style="text-align: right; font-size: small;">Minimum Documentation Searched <sup>7</sup></div> <div style="display: flex; justify-content: space-between; font-size: small;"> <span>Classification System <sup>1</sup></span> <span>Classification Symbols</span> </div> <div style="padding: 10px 0;">             IPC<sup>5</sup>      C 12 N, A 61 K, C 07 K           </div> <div style="text-align: center; font-size: x-small; margin-top: 10px;">             Documentation Searched other than Minimum Documentation<br/>             to the extent that such Documents are included in the Fields Searched <sup>8</sup> </div>   |  |  |
| <b>III. DOCUMENTS CONSIDERED TO BE RELEVANT</b> <sup>9</sup>   |  |  |
| Category <sup>9</sup>  | Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup>   | Relevant to Claim No. <sup>13</sup>  |
| X  | WO, A, 88/06626 (WHITEHEAD INSTITUTE FOR<br>BIOMEDICAL RESEARCH)<br>7 September 1988<br>see page 8, lines 13-21; page 15,<br>lines 17-23; claims<br>--   | 1-3,6  |
| X  | Proc. Natl. Acad. Sci. USA, volume 85,<br>September 1988,<br>S.B. Snapper et al.: "Lysogeny and<br>transformation in mycobacteria: Stable<br>expression of foreign genes",<br>pages 6987-6991<br>see the whole article<br>-- | 1-3,6  |
| P,X  | WO, A, 90/00594 (WHITEHEAD INSTITUTE FOR<br>MEDICAL RES.)<br>25 January 1990<br>see page 20, lines 21-29; page 42,<br>line 1 - page 43, line 26; page 56,<br>line 17 - page 62, line 9; figures<br>15-16; claims<br>--       | 1-3,6  |
| <div style="display: flex; justify-content: space-between; font-size: x-small;"> <div style="width: 45%;"> <p><sup>10</sup> Special categories of cited documents:</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubt on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</p> <p>"Z" document member of the same patent family</p> </div> </div> |  |  |
| <b>IV. CERTIFICATION</b>   |  |  |
| Date of the Actual Completion of the International Search<br><div style="text-align: center; font-size: large;">19th September 1990</div>  |  | Date of Mailing of this International Search Report<br><div style="text-align: center; font-size: large;">24. 10. 90</div> |
| International Searching Authority<br><div style="text-align: center; font-weight: bold;">EUROPEAN PATENT OFFICE</div>  |  | Signature of Authorized Officer<br><div style="text-align: center;"> <br/> <b>M. SOTELC</b> </div>                         |

## FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

P, X

Journal of Bacteriology, volume 172,  
no. 2, February 1990, American  
Society for Microbiology,  
R.N. Husson et al.: "Gene replacement  
and expression of foreign DNA in  
mycobacteria", pages 519-524  
see the whole article

1-8, 10, 15-20

V. ☒ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE <sup>1</sup>

This International search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☒ Claim numbers ..... \* ..... because they relate to subject matter not required to be searched by this Authority, namely:

\* - claims 12-14, 21-22  
see PCT - Rule 39.1(IV) methods for treatment of the  
human or animal body by surgery or therapy, as well as  
diagnostic methods.

2. ☐ Claim numbers ..... because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claim numbers ..... because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING <sup>2</sup>

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

## Remark on Protest

- ☐ The additional search fees were accompanied by applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

ANNEX TO THE INTERNATIONAL SEARCH REPORT  
ON INTERNATIONAL PATENT APPLICATION NO.

US 9003451

SA 38121

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 03/10/90. The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

| Patent document<br>cited in search report | Publication<br>date | Patent family<br>member(s) | Publication<br>date |
|---|---------------------|----------------------------|---------------------|
| WO-A- 8806626                             | 07-09-88            | EP-A- 0347425              | 27-12-89            |
| WO-A- 9000594                             | 25-01-90            | AU-A- 3867789              | 05-02-90            |

